Using Proteomics Approaches to Understand Mechanisms Underlying Low LET or GCR Radiation-Induced Cardiovascular Disease

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Abstract

The Earth's atmosphere and magnetic fields protect living organisms from most space radiation. Therefore, the effects of the unfiltered space radiation environment on biological systems remain poorly understood. Because astronauts journeying beyond low Earth orbit (LEO) will be continually exposed to the space radiation environment for periods as long as three years, it has become essential that the effects of space radiation type and quantity on health be more thoroughly studied, with one of the areas of focus being the cardiovascular system. Extrapolation of data from studies of terrestrial radiation exposure and astronauts' exposure has still not clarified whether cardiovascular damage from low dose galactic cosmic radiation (GCR) is a real concern, or whether the extrapolation of effects from terrestrial radiation to GCR is even valid. Furthermore, the major types of cardiovascular dysfunction arising from this long-term exposure to the space radiation environment and the molecular mechanisms that contribute to this damage are still unclear. It is evident that many unanswered questions remain in this field of study. While the use of 'omic technologies (genomics, transcriptomics, epigenomics, metabolomics, lipidomics etc.) can provide information on these mechanisms of radiation injury and repair, this review highlights the use of proteomics as a means to establish a functional view of real-time radiative effects, which can be integrated with these other "-omics" techniques. This review suggests that study of the proteome may provide a means to understanding the mechanisms responsible for low linear energy transfer (LET)

radiation-induced cardiovascular disease (CVD), which may in turn help to answer many of these unresolved questions for GCR.

Radiation-Induced Cardiovascular Damage

The topic of space radiation-induced cardiovascular damage has been recently reviewed by Boerma *et al.* [1]. The space environment exposes astronauts to chronic, low dose radiation in the form of a complex mixture of radiation including gamma, high energy proton, and heavy ion radiation [1, 2]. The majority of data on radiation-induced CVD originates from studies of terrestrial radiation exposure (atomic bomb survivors, nuclear accidents, or patients undergoing medical treatments) [3]. From these studies, which generally focus on mortality after irradiation, myocardial fibrosis and ischemic heart disease predominate as being associated with both high and low levels of radiation [3, 4]. While these epidemiological studies of terrestrial radiation exposure suggest that these types of injury will also occur from space radiation exposure, there is a high degree of uncertainty since space radiation is very different from the terrestrial radiation types that have been typically studied. Radiation in deep space comes in a mixture of many radiation types, including heavy ion radiation, which transfers energy to tissue differently than the gamma radiation more normally experienced terrestrially. Heavy ions deliver more energy as the particles slow in tissue, thus causing greater injury to deeper tissues selectively [5]. As a result, GCR is thought to have biological effects different from commonly encountered terrestrial sources such as X-rays and gamma rays. This limitation to relating low LET biological effects to those induced by GCR has been illustrated in unexpected changes in the central nervous system after exposure to GCR, in the form of altered genes and protein expression [6]. These findings demonstrate that biological effective dose received by organisms in the space radiation environment is much higher (and potentially more detrimental) than that generally observed terrestrially (see Table 1). Furthermore, living organisms have adopted a number of mechanisms to counteract the effect of low dose LET exposure; however, Tsuruoka

et al. have demonstrated that these repair mechanisms may be ineffective against GCR [7]. This difference in radiation type necessitates experiments which utilize a more representative radiation environment to that which will be experienced by astronauts traveling beyond low earth orbit (LEO).

Table 1: Radiation dose equivalents (mSv) under varying circumstance [8, 9].

	Dose Equivalent (mSv)
Annual Cosmic Radiation (sea level)	approximately 0.3 [4]
Radon in Average U.S. Home	2.28 [4]
Public Annual Limit	5 [5]
Whole body CT (single procedure)	10 [4]
Apollo 14 (9-day mission to the Moon)	11.4 [5]
6 Months on International Space Station	160 [5]
Estimated Mars Mission (3 years)	1,200 [5]

Currently only 24 astronauts have traveled beyond LEO. A higher rate of CVD has been observed in these individuals, but due to the small sample size it is difficult to make definitive statements as to the causal effect of space radiation on the cardiovascular system [10]. In addition, there are numerous confounding factors that obscure an understanding of the relationship between space radiation and CVD, such as the microgravity environment, varying ages and genders of the astronauts, and a multitude of other physical, mental, and social stressors involved in space travel. Correspondingly, because many symptoms of CVD are only apparent after long periods of time, it is difficult to distinguish whether these symptoms are a direct result of space radiation, or due to other factors such as astronaut genetics or lifestyle post-space travel.

It is also important to note that many of the body's responses to radiation are tissue specific, and therefore non-cardiovascular tissue may exhibit responses to radiation that are non-representative of

cardiovascular tissue, and potentially misleading. It could be that terminally differentiated tissue types, such as cardiovascular tissue and nerve tissue, will respond differently to GCR exposure than undifferentiated tissues, such as stem cells or progenitor cells. Therefore, in the consideration of CVD, study of cardiovascular tissue specifically must be performed to avoid this potential for confusion.

Because of these confounding factors inherent in epidemiological studies, experimental approaches become necessary for clarification of radiative effects. By using animal and cell models, and treatment with heavy ion or proton radiation, some of these confounding factors can be neutralized. However, even in these controlled situations the wide variability between symptomatic effects at the organ, organ system, and organismal level render many functional assays uninformative on their own. According to Tapio *et al*., this is where the mechanistic focus of proteomics can prove useful [11]. By circumventing symptom measurement, and instead identifying and quantifying damage pathways at the cellular and molecular levels, proteomics offer unique information to help answer the unresolved questions of the adverse effects of space radiation on the cardiovascular system. Proteomic analyses are especially useful when integrated with functional assays, such as echocardiography and calcium scoring, to provide a more holistic understanding of radiation injury to the heart.

Current "-omics" For Damage Assessment and the Proteome

The central dogma of molecular biology states that "… the coded genetic information hard-wired into DNA is transcribed into individual transportable cassettes, composed of messenger RNA (mRNA); each mRNA cassette contains the program for synthesis of a particular protein (or small number of proteins)" [12] (Figure 1; DNA → mRNA → protein). Proteins produced from genetic material are ultimately responsible for carrying out the majority of cellular functions. Adverse effects of radiation at the cellular and molecular levels have long been characterized by changes in DNA through the field of study collectively referred to as genomics. These studies of the genome, which contains the instructions

and information the cell needs (Fig. 1), have illuminated important radiative effects. For example, studies on both low LET [13] and heavy ion exposure [14] have mapped chromosomal structure and function following radiation exposure to provide insight into the repair mechanisms in place to counteract radiation-induced genetic damage, such as double-strand breaks and other genomic instability. Epigenomics, or studies of the modifications on the genome at a given time, can also be informative. For example, in a study on radiation-induced cognitive impairment, Acharya et al. have identified increased methylation of DNA as an effect of radiation [6]. They were able to subsequently inhibit this methylation by treatment with 5-iodotubercidin, and thus restored behavioral performance to previously cognitively impaired animals. Similarly, study of the transcriptome, or the set of RNA transcripts containing the message for generating proteins, has also revealed modulation of gene expression after radiation exposure [15]. However, mRNA is generally transient, typically having less than a 10 hour half-life (Fig. 1). Due to this transiency, it is uncertain that transcriptomics will be entirely effective for identifying long-lasting effects. A more complete view of radiation-induced damage on the cardiovascular system might be obtained by integrating these approaches with a study of proteomic changes.

The proteome is the set of proteins in a cell at any given time, and this complete set of proteins performs many tasks in the cell. Proteins are responsible for controlling cell structure and integrity, causing motion at both the intracellular and organismal levels, regulating processes such as transcription and translation, and protecting DNA, to name just a few of their many functions. In general, proteins are more stable than mRNA transcripts, having on average a 50 hour half-life in humans (Fig. 1). An analysis of these “working class” molecules can provide unique information into the current functionality of the cell. Whereas each cell of an organism has the same, seemingly static genome, different cells in the body contain a wide variety of proteomes [16]. The proteome can change depending on cell type, environmental stressors, and other cellular needs. For example, the proteome is responsible for ultimately causing an endothelial cell to behave differently than a cardiac muscle cell. These characteristics

demonstrate the strengths of integrating studies of the proteome with other '-omics' for more complete understanding of the mechanisms behind radiation-induced CVD.

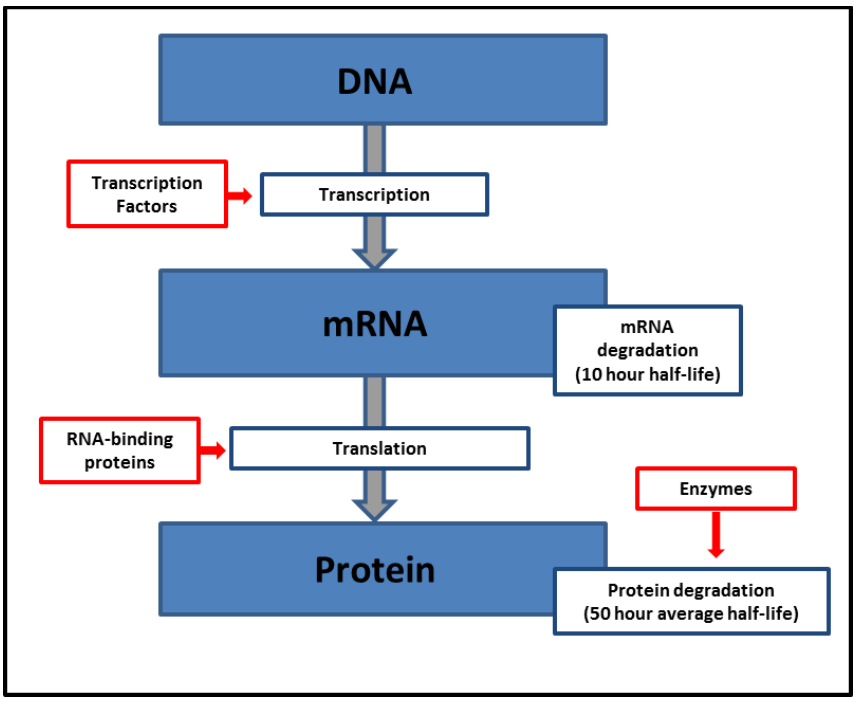


Figure 1: The Central Dogma of Molecular Biology, illustrating how proteins are involved at every level of cellular function, and are more stable than mRNA.

The term proteomics was only recently coined (1997), and the use of techniques and development of guidelines to study the proteome is still maturing [17]. It has only been in the past ten years that proteomic studies have been undertaken to better understand radiation biology. Indeed, the few studies exploring proteomic changes in the cardiovascular system after radiation already allude to definite mechanistic trends. The field of proteomics has already proven to be a valuable tool in deepening the science community's knowledge of the impact of radiation on cardiovascular health, but there is still much room for growth.

Types of Proteomics

There are a wide range of technologies utilized in proteomic studies, each with different strengths and focuses. While it is beyond the scope of this review to delve into much detail, what must be emphasized is that all of these technologies rely upon fundamental characteristics of proteins to identify and/or quantify particular proteins of the proteome. Proteins, and the peptides that result after protein digestion, each have a unique set of characteristics that can be matched to previously established identities in protein databases. Some of these characteristics include the proteins' or peptides' mass to charge (m/z) ratio, and their stability as ions [18]. Mass spectrometry (MS) is commonly utilized to identify these characteristics. The technique of MS consists of three main processes [18]. First, the sample is injected and prepared, generally by being vaporized and ionized, to allow for m/z measurement. Second, the mass analyzer component of the spectrometer analyzes the sample by separating the different proteins based on unique characteristics such as m/z ratio, ion stability, and the time required for the sample to pass through a chamber (time of flight). Lastly, the resulting data output is processed and compared to information already compiled in protein databases, such as PeptideAtlas [19] or the PRIDE (Proteomics Identifications) Archive [20], matching the readings from the mass analyzer to the corresponding protein or peptide. Because of the importance of matching to known databases in this final identification step, it is important that protein databases are well-curated and kept up to date. Identified proteins can then be quantified relatively through label-free techniques such as spectral counting and the comparing of peak intensity [21]. Other approaches for quantitation include labeling (described later in the dynamic proteomics section) with, for example, heavy isotopes to produce peak pairs which can be compared and quantified [22].

There are many different variations on the process of MS proteomics, and other steps which can be incorporated to improve the sensitivity and accuracy of the readings. Yates et al. detail some of the

most common analyzers used, including Ion Trap, Orbitrap, and ion cyclotron resonance (ICR) instruments, comparing their analysis techniques and their utility in different types of proteomics [18]. Sample preparation protocols are also numerous, as well as ionization techniques.

Another common technique widely used in proteomics is 2D-gel electrophoresis (2DE), which can be used as a stand-alone method of analysis or can be used to separate proteins before mass spectrometry is performed (Figure 1A). 2D-gel electrophoresis uses two properties of proteins (charge and size) to achieve separation while they are placed in an electric field. In the first dimension (horizontal) proteins in a mixture are separated according to their isoelectric point (pI); in the second dimension (vertical) these proteins are further separated according to molecular mass. 2DE can be performed using labeled or unlabeled protein samples, to help identify proteins in the gel. The intensity of the spots obtained in the 2D gel illustrate the relative abundance of the particular protein in a sample, whereas the size, or spread, of the spot may relate to the amount of permutations of PTMs on a protein (all with nearly the same molecular mass and pI as the original protein).

After separation by gel electrophoresis, an in-gel digestion of the proteins can be performed, and the resulting peptides can be extracted for identification via mass spectrometry. It is important to note that this additional manipulation can introduce contaminants to the sample, and thus is less effective when especially sensitive measurements or high quality samples are needed (such as in post-translational modification studies) [23].

As previously mentioned, these fundamentals of proteomic technologies are employed in many variations to obtain different information. In this review, we have categorized different types of proteomic studies into four areas, as follows.

Unbiased: In studies that perform unbiased proteomics, researchers attempt to simultaneously examine a wide array of undefined proteins and compare abundances under different conditions.

Sometimes referred to as "shotgun proteomics", these studies survey the entire proteome without initial selection or separation. Typically, technologies for unbiased proteomics include either mass spectrometry, two-dimensional gel electrophoresis, or both, to identify and characterize abundance of whole proteins or fragments. There are two general methodologies these studies can employ. First, Bottom-up Processing refers to digesting the extracted proteins with an enzyme such as trypsin, and analyzing the resulting peptide fragments using mass spectrometry. This is the more common methodology used, because peptide separation is easier than protein separation, and the separated peptides can be quantified with high sensitivity [18]. In contrast, Top-down Processing analyzes an entire, intact protein, allowing for better visualization of post-translational modifications (PTMs) and higher sequence coverage. State of the art mass spectrometry even allows for multiple PTMs to be examined on the same peptide chain using top-down processing, which enables a more complete understanding of the combined effect of the multiple PTMs on the peptide [24]. Benefits of unbiased approaches include the large number of proteins detected, while limitations include that proteins present in lower amounts may be overlooked.

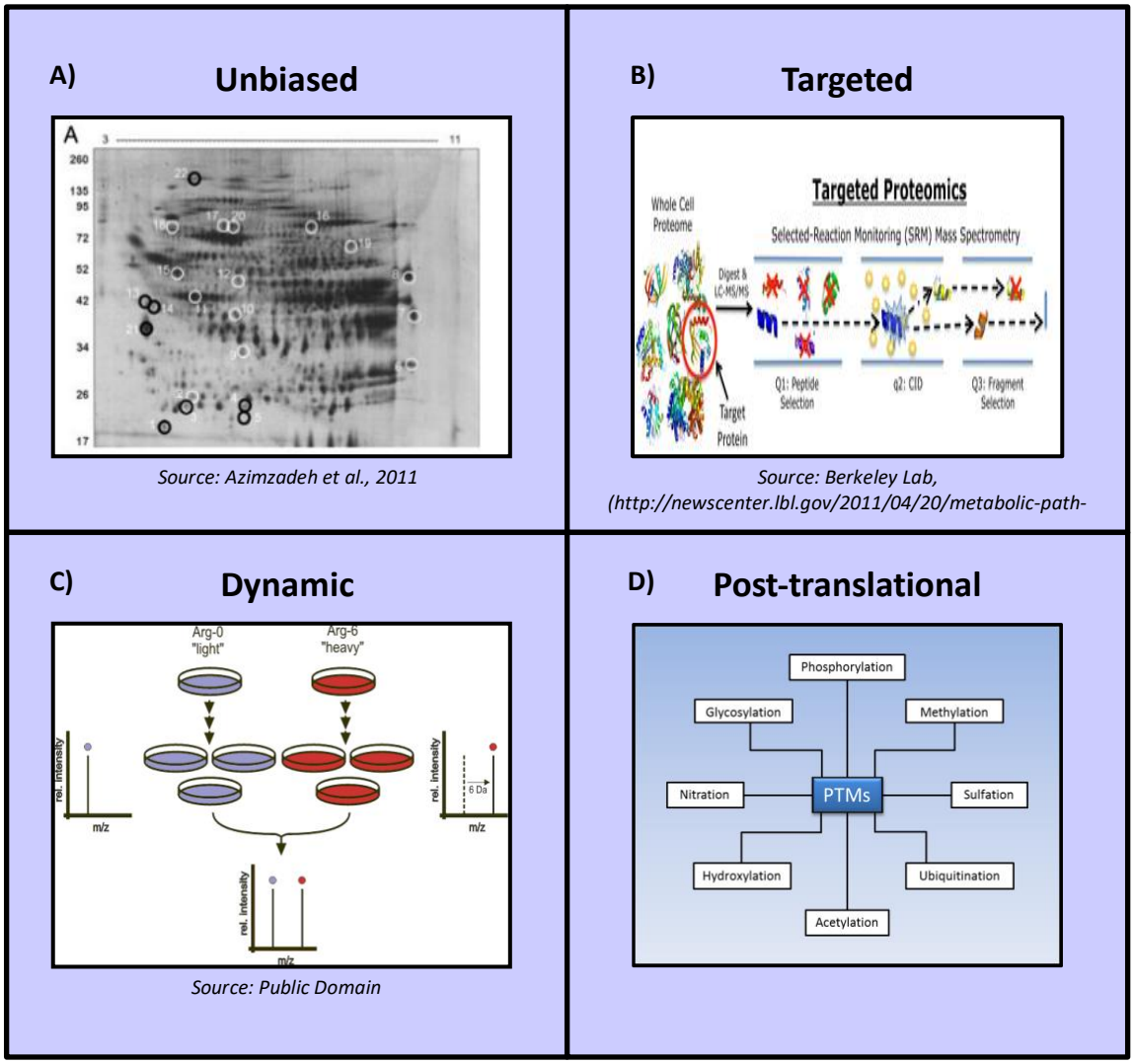
Targeted: Using targeted proteomics approaches, investigators focus their examination on a single protein or on pre-defined groups of proteins. This eliminates the need to process, format, clean, and statistically and bioinformatically analyze the enormous amount of data generally returned from unbiased approaches. Techniques used in targeted proteomics can include western blot analyses [11], which use antibodies to detect specific proteins, or mass spectrometry (MS) approaches such as selected-reaction monitoring (SRM) or multiple-reaction monitoring (MRM) in which antibodies are not required [25]. Instead, MS/SRM uses chemical labeling and the m/z values of distinct precursor and product fragment ions of a protein to identify and/or quantify the

protein of interest [26]. Lange et al. describe this procedure in great depth [27]. The precursor peptide of interest is first selected. A heavy labeled synthesized copy of this peptide can then be used to identify which fragments (or reactions) can be expected to take place. The peptide of interest is then either fragmented physically or by simulation (*in silico*). The distinct product fragments which result are then monitored to obtain a very specific quantification. As long as the protein is present in high enough levels, this technique has been found to be extremely fast and accurate. Limitations to this technique are the inability to detect low concentration levels, as well as the relative complexity of selecting fragment ions and the subsequent analysis involved.

Dynamic: Dynamic proteomics are used to monitor protein synthesis and protein degradation occurring in cells. Depending upon the type of analysis one can determine rates of synthesis or degradation over time, or the analysis can provide a snapshot of what is being synthesized in a narrow window of time. Important for dynamic proteomics is that proteins are labeled during translation by incorporating synthetic or pre-labeled amino acids. One commonly utilized technique is Stable Isotope Labelling with Amino acids in Cell culture (SILAC), which incorporates an amino acid analog such as heavy arginine into newly synthesized proteins [28]. The ratio of the newly labeled, heavy protein to the native, light protein is used to calculate the relative isotope abundance, thus determining the amount of protein turnover actively occurring at the time points examined [29]. This chemical labeling approach has also been expanded to use in rodent models, in a process called Stable Isotope Labelling with Amino acids in Mammals (SILAM). This is done by preparing rodent chow that replaces canonical amino acids with identifiable isotopes, such as azidohomoalanine (AHA) in the place of methionine. In this case, the azido group of the newly incorporated AHA can be biotinylated in a simple reaction, thus allowing for easy identification of newly synthesized proteins. This ability to label animal models for protein quantification and

identification of newly synthesized proteins is especially relevant to studies of radiation-induced CVD. By AHA feeding post-irradiation, changes in protein levels as a result of radiation can be examined, providing a "snapshot" of the proteome at that time-point that other proteomic techniques could otherwise miss.

Post-translational: A protein will often undergo post-translational modifications (PTMs) to signify its maturity, target it, or change its functionality. For example, a phosphate group added to a protein (phosphorylation) can confer new functionality upon it, or activate it to perform various functions [30]. An added carbohydrate (glycosylation) can initiate the folding of the protein [30], or be involved with cell-cell adhesion [31]. Ubiquitination can mark the protein for degradation [30]. These modifications can be identified by proteomic techniques such as mass spectrometry, by matching the expected mass shift in the spectrometer readout to the corresponding PTM and parent peptide. As mentioned previously, MS techniques are preferred to gels in PTM studies because they introduce fewer contaminants. In addition, there are multiple possible PTM that may occur on a single protein. As a result each gel spot may contain a complex mixture of proteins that may not be sufficiently separated using a 2D gel. This branch of proteomics has already demonstrated its utility in regards to radiation-induced injury. One PTM clearly associated with radiation exposure is carbonylation, resulting from oxidative stress and the increased reactive oxygen species present [32, 33]. Studies of PTMS have also shown acetylation of certain proteins to be caused by radiation. This insight has identified these modified proteins' involved pathways as areas of interest for future radiation research [34].



Source: Azimzadeh et al., 2011

Source: Berkeley Lab, (http://newscenter.lbl.gov/2011/04/20/metabolic-path-

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Figure 2: Illustration and examples depicting the four different proteomic approaches discussed. **A)** A 2D gel is shown under the unbiased section, showing how all proteins can be separated and then extracted for later identification by mass spectrometry. Each circle highlights a protein complex which has been separated by pI (x axis) and molecular mass (y axis). **B)** An MS targeted proteomics approach, selected-reaction monitoring (SRM). Like MRM, which performs this for multiple targets, this technique quantifies a protein of interest by quantifying a specified peptide fragment after protein digestion and fragmentation. **C)** A diagram of SILAC, where bacteria have been grown in both media containing native arginine (light) and in media containing a heavy isotope of arginine (heavy). These bacteria are grown, incorporating their respective arginine isotopes, and then mixed and analyzed. The resulting ratio is used to determine the protein turnover in the sample. **D)** A list of common PTMs which can be examined by various proteomics techniques.

There is often overlap between these categories of proteomics. Many studies will perform an unbiased scan of the proteome first, quantifying altered protein levels, before focusing in on one pathway

or a single protein which they wish to study further. This strategy combines both the unbiased and targeted approaches, discovering proteins of interest and then validating findings, and these combinatorial strategies provide additional insight into the mechanisms behind phenotypic changes.

Another example of a combinational approach involves selecting for proteins with a post-translational modification (PTM), performing MS assessment on this enriched sample, and then comparing data obtained to replica samples which underwent a MS assessment but without the selection. Schechter *et al.* utilized this type of combinatorial approach by selecting from homogenized cardiac tissue samples proteins which were phosphorylated using a TiO_2 column and then comparing the proteins that had been phosphorylated to samples that had not undergone the TiO_2 selection [35]. This study demonstrated that while quantification of levels of individual proteins in the unbiased proteome did not differ between non-ischemic and ischemic heart disease, the site-specific phosphorylation status of particular proteins was a potentially important distinction between different etiologies of heart failure.

Current knowledge of proteomics/radiation-induced CVD

A systematic search of the PubMed, Embase, and Ovid databases for “Proteomics” AND “Radiation” AND “Heart” was performed, which returned 98 articles. After thorough review of these articles by two separate readers, 27 articles were chosen as most relevant to this topical review of proteomic analysis of radiation-induced CVD (Table 2). Due to tissue specific radiative effects as previously detailed, this literature search was designed to focus on the effects observed in heart tissue specifically. Also, the majority of these articles utilized low LET radiation rather than simulated GCR, which is an important shortcoming of the current literature. It must be noted that the effects of low LET radiation on the heart environment may be different from those caused by GCR, and this will be an important area for future study. However, the findings presented by these selected studies do demonstrate consistent

biological effects, which can be broadly categorized into three observed effects: mitochondrial, oxidative stress, and structural.

Table 2. Summary of literature categorized into three major topic areas.

	Mitochondrial Dysfunction	Oxidative stress response	Cytoskeletal Deregulation
Author Year(s) [Reference]	• Azimzadeh 2011, 2012, 2013, 2015, 2017 [4, 32, 36-38] • Klionsky 2016 [17] • Percy 2013 [25] • Sun 2016 [39] • Barjaktarovic 2011, 2013, 2015 [34, 40, 41] • Lai 2016 [42] • Liu 2004 [43] • Liu 2014 [44]	• Azimzadeh 2011, 2012, 2013, 2015, 2017 (2) [4, 32, 36-38, 45] • Klionsky 2016 [17] • Percy 2013 [25] • Barjaktarovic 2015 [34] • Liu 2014 [44] • Butterfield 2014 [46] • Zhao 2014 [47] • Tapio 2013, 2017 [33, 48]	• Lai 2016 [42] • Butterfield 2014 [46] • Tapio 2013, 2017 [33, 48] • Subramanian 2017 [49] • Bakshi 2013, 2016 [50, 51]

First, the largest number of papers described proteomic changes consistent with loss of mitochondrial function at both high and low IR exposure [4, 17, 25, 32, 34, 36-44]. This was shown by identifying decreased levels of mitochondrial-related proteins in radiation-treated experimental groups as compared to control groups. Azimzadeh *et al*. demonstrate decreased levels of mitochondrial complexes I, III, and IV in response to irradiation, resulting in impaired mitochondrial respiration, as well as an increase in oxidative stress [38]. Barjaktarovic *et al*. go even further, identifying the APOE protein as being necessary to this "impairment of the mitochondrial respiration" [41]. They do this by studying both normal mice and APOE deficient mice after 2 Gray (Gy) irradiation, observing that the deregulation of mitochondrial complexes in the APOE deficient mice was less severe. They suggest that APOE has a direct interaction with mitochondrial complexes III and IV. These findings of mitochondrial disruption due to

APOE are not entirely unexpected; they have been previously demonstrated in studies on Alzheimer's disease (AD), which have identified APOE4 as disrupting mitochondrial respiration [52, 53].

The second widely reported adverse effect of radiation on the cardiovascular system was that of oxidative stress, which refers to an imbalance of free radicals and the inability for antioxidants to neutralize them [4, 17, 25, 32-34, 36, 37, 44-48]. This may be an indirect effect of the radiation, resulting from damaged mitochondrial complexes and processes which would normally defend against the formation of reactive oxygen species (ROS) and their toxicity. In at least two cases post-translational proteomics identified increased protein carbonylation, resulting from an increase in ROS, as evidence of oxidative stress [32, 33]. In five separate studies [4, 17, 25, 34, 37] oxidative stress is reported as a result of mitochondrial impairment, which in turn caused further mitochondrial impairment, forming a vicious cycle. Because the mitochondria are the site of oxidative phosphorylation, it is not surprising that the two concepts are related.

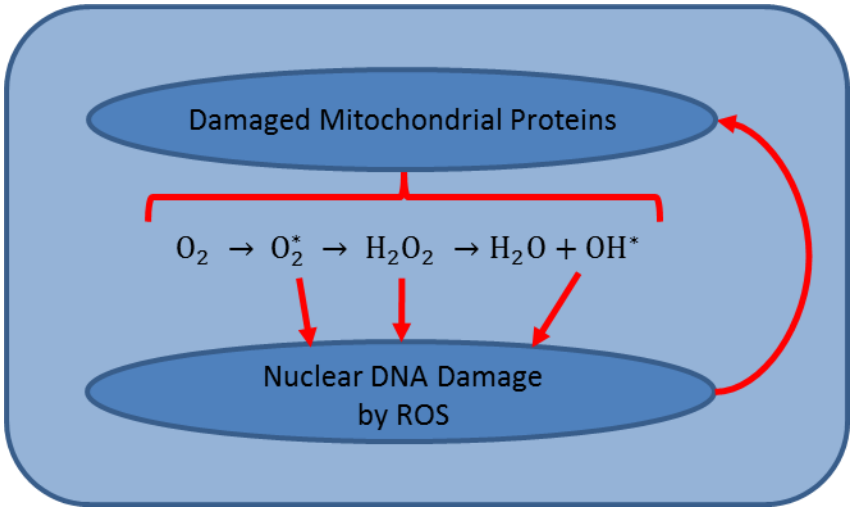

Figure 4: Cyclical effect of ROS production by mitochondrial damage.

The third effect of radiation emphasized by current literature was on cytoskeletal protein dysregulation, such as the down-regulation of myosin heavy chain, desmin, and vimentin [33, 42, 46, 48-

51]. These effects were predominantly reported in studies utilizing prenatal or neonatal samples [50, 51]. A reduction in cytoskeletal proteins (structural proteins) was noted even at doses as low as 0.5 Gy in younger samples. Doses of local radiation as high as 16 Gy in adult murine hearts caused TGF-beta to be up-regulated, which caused inflammation and fibrosis [49]. These studies demonstrate that the effects of radiation on the cytoskeleton are manifold, and related to exposure levels.

Mitochondrial impairment, oxidative stress, and cytoskeletal changes are the most widely observed effects of radiation currently identified through proteomics. However, these are by no means the only effects reported. For example, Pluder *et al*. report transient decreases in heat shock proteins, which are involved in stress response, as well as proteins involved in endoplasmic reticulum transport, which are related to protein synthesis [54]. Because heat shock protein function can often be determined by its PTMs, a proteomic PTM analysis could be valuable in this case. Furthermore, and unsurprisingly, many studies point to increased levels of pro-inflammatory [32, 55] and pro-fibrotic proteins [37, 49] resulting from radiation. Again, targeted proteomics of these pro-inflammatory and pro-fibrotic proteins could elucidate mechanisms by which their effects could be counteracted, and CVD avoided.

The most frequent changes to the proteome of cardiac tissue highlighted by current literature become extremely informative in the context of possible effects on overall heart health. The disruption of mitochondrial respiration, as well as increases in reactive oxygen species, have been shown to lead directly to cardiac hypertrophy and resulting heart failure [56]. The fact that mitochondrial impairment and oxidative stress are frequently observed post-low LET radiation suggests that hypertrophy may be one of the first pathologies to consider for study in post-GCR models. Similarly, cytoskeletal changes like those observed post-radiation have been shown to cause cardiac arrhythmias, and in the case of desmin deregulation, dilated cardiomyopathies [57]. Again, this provides insight into the pathologies that might be most prevalent post-GCR.

Summary and Future Directions

Due to fast-approaching Mars expeditions, there is increased interest in the effects of space radiation on organisms, especially the cardiovascular system. This review has identified mitochondrial damage and increased reactive oxygen species (ROS) as the over-arching effects which current literature shows are involved in low-LET radiation-induced CVD, especially from the proteomic perspective. The cytoskeleton and pro-fibrotic and pro-inflammatory pathways are also implicated and should be targeted in further study. This analysis illustrates the complex interactions and entwined nature of the affected cellular processes, and the added insight proteomics can provide in combination with other "-omics" and functional assays of the heart. This fully integrated approach will allow for mechanistic radiative effects to be understood, as well as their potential impact on overall cardiovascular health. Beyond clarification of cytoskeletal effects, future proteomics studies in the area of radiation should examine the linearity of the relationship between exposure levels and proteomic alterations. It may well be that below a certain threshold of radiation dose there are mechanisms in place to repair proteomic changes and avoid functional loss. However, few studies have provided more than two levels of radiation exposure. By comparing proteomic alterations at more exposure levels, the question of the linearity of this relationship may be resolved.

A limitation to current research is the paucity of information in regards to GCR-induced CVD. As illustrated by recent studies of the biological effects of GCR on the central nervous system, low LET induced changes may be poor indicators of GCR induced changes, even at the level of the proteome. This uncertainty of the validity of low LET to predict GCR effects will need to be addressed with further research employing simulated GCR radiation.

Proteomics will continue to play an important role in the understanding of the effects of space radiation on the cardiovascular system, especially in combination with functional assays and other "-

omics". As the field of proteomics continues to mature, additional mechanisms of radiation-induced damage to all organ systems may begin to emerge, helping to resolve the issues currently confounding the study of radiation-induced cardiovascular disease.

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